

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: RAMADN, Eric et al.
Title: METHOD FOR PRODUCING OLIGOPOLYSACCHARIDES
Appl. No.: 10/019,954
Filing Date: 5/24/2002
Examiner: Rebecca F. Prouty
Art Unit: 1652
Conf. No.: 6242

DECLARATION UNDER 37 C.F.R. § 1.132

Commissioner for Patents

Sir:

I, Eric Samain, declare as follows:

1. I am one of the inventors of the captioned application.
2. I am currently employed at Centre National de la Recherche Scientifique—
Centre de Recherches sur les Macromolécules Végétales ("CNRS-CBRMAV").
3. My academic background and work experience are summarized in my
curriculum vitae, which is attached as Exhibit A. Briefly, I did my PhD on the microbiology
of methanogenic fermentation in a laboratory of the French National Institute for
Agronomical Research (I.N.R.A.) located in Lille, France. I was recruited as a research
engineer in 1982 in the same laboratory to pursue my PhD work on the physiology of bacteria

involved in methanogenesis. I then worked on the bacterial production of glycosylhydrolases for agricultural uses and developed a patented fermentation process for the high yield production of a thermophilic xylanase. In 1990 I was offered a position at the CNRS (National Center for Scientific research) in Grenoble to develop new fermentation processes related to the degradation and the biosynthesis of carbohydrate in a CNRS institute (CFRMAV) which is considered as the most important European Research Institute devoted to the study of carbohydrates. Since 1995, my activities have been focused on the synthesis of oligosaccharides by metabolically engineered bacteria, and my research in this field has been recognized with the esteemed "Cristal of CNRS" awards in 2004, and it has earned me invitations to lecture at several international meetings. Over the years, I have produced more than 45 research papers in microbiology and biotechnology and two book chapters on microbial oligosaccharide production. I have submitted 4 patents in the area of oligosaccharide synthesis and have directed the research of several Ph.D. students and Master's students.

4. I have read and understand the Office Actions dated December 29, 2005, and January 9, 2008. Among other rejections, I understand that the 2008 Office Action rejects the claims as obvious over the following references, either alone or in combination:

(a) ("Koizumi et al.") Koizumi S., Enjo T., Tabata K. and Ozaki A. (1998) Large scale production of UDP-galactose and globotriose by coupling metabolically engineered bacteria, *Nature Biotechnol.* 16, 847-850

(b) ("Bettler et al.") Bettler E., Samain E., Chazalot V., Bosso C., Heyraud A., Jozeasse D.H., Wakarchuk W.W., Imberty A., Geremia R.A. (1999) The living factory: in

vivo production of N-acetylactosamine containing carbohydrates in *E. coli*, *Glycoconj.* 6(3):205-12.

(c) ("Dykhuizen et al.") Dykhuizen D., Hartl D. (1978) Transport by the lactose permease of *Escherichia coli* as the basis of Lactose killing, *Journal of Bacteriology*, 135, 876-882

(d) Ahmed S, Booth IR (1983) The effect of beta-galactosides on the proton motive force and growth of *Escherichia coli*, *J Gen Microbiol.* 129(8):2521-9.

5. The 2008 Office Action (page 4, lines 3-5) states that "claims 1, 5-7, 9-12, 27, 28, 39, 47 and 48 are rejected under 35 U.S.C. 103(a) as being unpatentable over Bettler et al. in view of Kozumi et al." The rejection is explained in the 2005 Office Action, which states that Bettler et al. "teach the intracellular production of the oligosaccharide Gal β -4(GlcNAc β -4) $_4$ (GlcNAc using a LacZ- *E. coli*" (2005 Office Action, page 8) and that Kozumi et al. "teach the production of the trisaccharide galactotriose from lactose using a permeabilized LacZ- *E. coli*" (2005 Office Action, page 9). The 2005 Office Action concludes (page 10) that "it would have been obvious to use the transformed LacZ- *E. coli* of Kozumi et al. without permeabilizing the membrane as taught by Bettler et al." and that "a skilled artisan would have been motivated to overexpress this gene [lactose permease] in the bacteria of Kozumi et al. as lactase is the precursor used by Kozumi et al." (2005 Office Action, page 10).

6. I submit that while Bettler et al. is part of the "March 1999 volume" of *Glycoconjugate Journal*, the print and release date of the "March 1999 volume" of *Glycoconjugate Journal* is actually September 24, 1999, as proved by the document attached as exhibit B from the Editor.

7. That means that the effective date on which Bettler et al. has been made accessible to the public is after September 24, 1999, and therefore after the priority date of the present application, which is July 7, 1999. As a result Bettler et al. cannot be considered prior art for the rejection of pending claims 1, 5-7, 9-12, 27, 28, 39, 47 and 48.

8. In response to the rejection of the claims under 35 U.S.C. 103(a) presented in §3 of the Office Action, we have stated in response to previous Office Actions that "one of skill would have no motivation to combine Koizumi et al. and Bettler et al., much less any expectation of success, because it was known in the art that rapid uptake of sugars by lactose permease disrupts membrane function . . . which results in growth inhibition and eventually cell death (i.e. 'lactose killing.'" Amendment filed February 28, 2007, page 14. Nonetheless, the 2008 Office Action states that "this is not persuasive because lactose killing as reported in [Dykhuizen et al.] is present in E. coli cells that have been growing on a limited supply of lactose when they were then provided with excess lactose but not in cells growing on other carbon sources when supplied with lactose". 2008 Office Action, page 4 (emphasis in original).

9. I agree with the fact that cells growing on other carbon sources are not affected by the lactose killing and this was clearly written (on page 877, lines 16-20) in Dykhuizen et al. However these E. coli cells growing on other carbon sources have of course no reason to be killed by lactose, because their lactose permease is not induced, since they have been grown in absence of lactose.

10. On the contrary as shown in table 2 of Dykhuizen et al., cultivation of *E. coli* cells on glucose or galactose in presence of IPTG (which is an inducer of the lactose permease) results in a strong lactose killing effect. The authors conclude (on page 878, column 2, line 11-17) that "there is strong correlation between the amount of lactose permease and the amount of lactose killing".

11. Thus, I state that the interpretation of Dykhuizen et al. by the Examiner contradicts this quoted statement within Dykhuizen et al. itself.

12. The 2008 Office Action also states that "the amount of growth inhibition produced by lactose can be diminished by reducing the rate of import of lactose into the cells and the presence of glucose or glycerol in the culture during the second phase of cell growth would do just that as they are well known to repress the lactose promoter" (2008 Office Action, page 5).

13. It is true that glucose (but not glycerol) represses the lactose promoter by a mechanism called catabolic repression. However, in the invention as claimed, the second phase of cell growth is carried out in carbon-limiting condition to precisely prevent this catabolic repression and enable the full expression of the lactose permease, which is a necessary condition for a very efficient system of oligosaccharide synthesis. One should keep in mind that the interest of this invention is its very high productivity and that we have later succeeded in obtaining by the process as claimed the production of complex oligosaccharides at a concentration of more than 25 g/l (see publication Fierfort and Samain, J. of Biotechnology 134 (2008) 261-263, in exhibit C).

14. A skilled artisan in this field would not have anticipated such excellent results. On the contrary, the skilled artisan would have considered that there was no industrial interest in developing a process whose yield would be limited by the lactose input due to the lactose killing effect. Thus, a skilled artisan would not have contemplated using a system as defined in the claims.

15. In addition, the lactose promoter and other catabolically repressed promoter such as the arabinose promoter are largely used in common expression vector and in particular in almost all the expression vectors that were used in the examples of the claimed invention to overexpress the genes for glycosyltransferase and other enzyme involved in sugar nucleotide biosynthesis that are required for the synthesis of complex oligosaccharides. Therefore, one skilled in the art would not have considered the process as claimed since partial repression by catabolic repression of the lactose promoter would affect not only the expression of the lactose permease but also the expression of other genes involved in oligosaccharide synthesis.

16. The 2008 Office Action also states, "Furthermore, a skilled artisan would be aware that even a low growth rate of the cells during the second phase could still be sufficient to produce large amount of the desired product" (2008 Office Action, page 5).

17. It is true that many products are produced in condition of low growth rate and this is actually the case of the claimed invention. However, the synthesis of oligosaccharide is an energy demanding process which requires metabolically active cells able to efficiently produce all the precursors such as the sugar nucleotides. The main fear that a skilled artisan

could have about the lactose killing is thus not the problem of slow growth but more the problem of irreparable damage to the cells, which would affect their metabolically activity and their energetic yield.

18. The strain used in reference Dykhuizen et al. is LacZ⁺. This designation means that cells as in Dykhuizen et al. hydrolyze and catabolize lactose.

19. In the claimed invention, the strain are LacZ⁻ and the lactose accumulates intracellularly at high concentration. A skilled artisan would have feared that this accumulation could be detrimental for the cells by dramatically increasing the intracellular osmotic pressure (turgor). This increase in turgor can cause cell death because of membrane rupture, and bacterial cells are known to adapt to severe turgor increase by opening stretch activated channel to let small molecule exit. As a small molecule lactose is likely to exit through the activated channel and to create an energy consuming futile cycle by being reincubated by the lactose permease.

20. I was the first to report and demonstrate that it is possible to maintain, for several hours and in an excellent state of metabolic activity, a high cell density population of E. coli cells that contain a high intracellular concentration of lactose (a metabolically active cell being defined as a cell that is capable to maintain its cellular integrity and to fulfill all the physiological functions of a living cell, e.g., protein and other macromolecule synthesis, ATP generation, and active transport). This condition is a prerequisite for the claimed invention because a metabolically active cell can express a recombinant glycosyltransferase, recycle

sugar nucleotides, and therefore glycosylate intracellular lactose to obtain the desired oligosaccharide.

21. However, as the accumulation of any metabolite is susceptible to be toxic for a cell, and the lactose killing effect is a well-known phenomenon, this prerequisite would not have been obvious for a microbial physiologist. Consequently, a microbial physiologist would not have been motivated to develop a system of oligosaccharide synthesis from lactose by living *E. coli*, considering the fact that Koizumi et al. and Bettler et al. described efficient systems, and a skilled artisan would have been more motivated to improve either the Koizumi et al. or the Bettler et al. process.

22. I hereby declare that all the statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements are made with the knowledge that willful false statements are so made punishable by fine or imprisonment, or both, under Section 101 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 9 July 2008 By: Eric Samarin
Eric Samarin